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TECHNICAL REPORT
FD-18

STUDY OF FAT OXIDATION PRODUCTS WITH OTHER FOOD COMPONENTS, ANTIOXIDANTS AND TRAPPING AGENTS TO INHIBIT CROSS-LINKING OF PROTEINS

by

FRED A. ANDREWS, DAVID A. THOMSON, CHESTER E. UNDERWOOD

AUGUST 1965

BJORKSTEN RESEARCH LABORATORIES, INC.
Madison, Wisconsin

Contract No DA19-129-AMC-71(N)

U. S. ARMY NATICK LABORATORIES
Natick, Massachusetts

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Natick, Massachusetts 01762

FOREWORD

Oxidative changes, especially those involving fats, remain a major cause of reduced acceptance after storage of ration items. As a part of a continuing research program on the mechanisms of deteriorative changes, model systems simulating dehydrated foods were developed. These model systems have been used to study the effects of a number of non-fat food components on the course of fat autoxidation.

The work covered in this report was performed by Bjorksten Research Laboratories, Inc. under Contract DA 19-129-AMC-71(N) during the period of March 1963 to March 1965. It represents a continuation of an earlier study in which it was found that certain of the oxidation products of unsaturated fatty acid caused a cross-linking in protein which could be inhibited by the addition of mannose. In this study other carbohydrates were investigated for their antioxidant properties in dehydrated model systems.

Fred A. Andrews was the official investigator for Bjorksten Research Laboratories, Inc. and David A. Thomson and Chester E. Underwood were his collaborators. The U. S. Army Natick Laboratories Project Officer was A. S. Henick and the Alternate Project Officer was M. C. Brockmann, both of Food Division.

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ABSTRACT

A number of aldose sugars and ribose derivatives have been studied manometrically and spectrophotometrically to assess their potentials as antioxidants in dehydrated foods.

Evidence is presented which indicates that aldoses containing cis hydroxyl configuration on carbons 2 and 3 of the molecule possess antioxidant properties. Ribitol, D-2-deoxyribose and riboflavin have also been found to inhibit peroxidation of purified methyl linoleate.

Ribose at very low concentrations has been found to inhibit diene conjugation in methyl linoleate.

Bjorksten Research Laboratories, Inc.

STUDY OF FAT OXIDATION PRODUCTS WITH OTHER FOOD COMPONENTS, ANTIOXIDANTS AND TRAPPING AGENTS TO INHIBIT CROSS-LINKING OF PROTEINS

INTRODUCTION

Previous studies in this Laboratory under Contracts No. DA 19-129-QM-1945 and DA 19-129-QM-1549 had shown that the principal interaction of model proteins with autoxidizing lipid in dehydrated systems was a cross-linking reaction. Through the use of Sanger's 1-fluoro-2, 4-dinitrobenzene technique, trypsin assays and hydrogen fluoride solubility tests, it was determined that the free amino groups of purified proteins were reacted with aldehyde intermediate oxidation products of methyl linoleate (ML). Further, it was noted that impure egg white was somehow resistant to the cross-linking effect of autoxidizing methyl linoleate. Since crude egg white is mposed primarily of ovalbumin and ovomucoid, both of which contain mannose, this sugar was studied for its antioxidant potential. Using dried gelatin-methyl linoleate foams containing mannose, it was found that the cross-linking interaction was inhibited.

In the present program the observations made on mannose have been broadened to determine whether other carbohydrates might be found which possess antioxidant properties of potential usefulness in prolonging the stability of dehydrated meats.

EXPERIMENTAL

A. Materials

Gelatin (310 Bloom pigskin) obtained from Grayslake Gelatin Co., Grayslake, Illinois, was used exclusively as a purified protein in foam studies.

Hanging beef tenderloin from U.S. Choice cattle was obtained one hour after slaughter from Oscar Mayer & Company, Madison, Wisconsin, while pork Longissimus dorsi and chicken muscle were obtained from a local meat market.

Methyl linoleate obtained from the Hormel Institute, Austin, Minnesota, was used throughout as a model lipid. D(+)Mannose, D(-)glucose, D(-)fructose, D(-)arabinose, D(-)ribose, D-xylose, D(-)lyxose, mannitol and inositol were obtained from Pfanstiehl Laboratories, Inc., Waukegan, Illinois. Other sugars and biochemical compounds were obtained from Nutritional Biochemicals Corporation, and all reagents grade chemicals were obtained from Mallinckrodt Chemical Works, St. Louis, Missouri.

B. Methods

1. Dehydrated Protein Models

Five-gram samples of gelatin were dissolved in 100 ml. of distilled water containing 500 mg. test antioxidant. The solutions were whipped to a stiff foam in a Sunbeam Mixmaster, and 1 gm. of methyl linoleate was added during the whipping step. Control foams (without ML) were prepared in a similar fashion for each test. The foams were shell-frozen and freeze-dried, and then incubated for five days at 50°C before being enzymatically hydrolyzed.

One hundred gram samples of hanging beef tenderloin, pork L. dorsi and chicken muscle (both white and dark), after removal of gross surface fat and connective tissue, were blended with 250 mg. of ribose in sufficient water to give smooth homogenates. Control homogenates were prepared in the same manner using 250 mg. of dextrose. The homogenates were then shell-frozen, freeze-dried and incubated at 50°C for five days.

Both the incubated gelatin and meat samples were extracted for 16 hours with benzene-ethanol azeotrope before being further analyzed.

2. Enzyme Hydrolysis Procedure

Samples of defatted foams (0.5 g.) were mixed in 125 ml. Erlenmeyer flasks with 60 ml. of Sörensen's buffer (pH 7.7) containing 10 mg. of Difco trypsin (1:250). Each sample was set up in duplicate with one being precipitated immediately upon mixing with 20 ml. of 20% trichloroacetic acid (TCA). The second flask was shaken for 24 hours at 37.5°C and then precipitated with 20 ml. of 20% TCA. Both samples were filtered and washed with 5% TCA to adjust the volume of the filtrate to 100 ml. The degree of hydrolysis was determined on the filtrates using the standard Van Slyke method for liberation of α-amino nitrogen.

Autoxidized meat samples were digested in similar fashion except that one-gram samples of meat with 40 mg. of trypsin were used in each digestion flask. In one series of tests the meat, suspended in buffer, was autoclaved for 15 minutes at 120°C, prior to the addition of the enzyme.

3. Measurement of Oxygen Absorption

In the manometric screening studies of various sugars and derivatives 3×10^{-4} moles of the test sugar were mixed directly with 6×10^{-4} moles of methyl linoleate in Warburg flasks. Oxygen consumption was followed at a temperature of 50°C with mercury in the manometers to insure anhydrous conditions. Manometer readings were taken at 15-minute intervals over an eight-hour period and the flasks were not shaken during reaction.

4. Diene Conjugation Studies

Ultraviolet absorption of diene conjugation at 231.5 mμ was used to study the induction times of methyl linoleate under oxidizing conditions. To compare antioxidant potency, flasks containing 10^{-2} μM of methyl linoleate in methyl alcohol were treated with ribose, vitamin E and butylated hydroxyanisole (.01%/mole of ML). After removal of methanol in vacuo over night, the flasks were placed in a 50°C oven. At the end of seven hours the flasks were removed from the oven and 40 ml. of methanol added to each to quench the reaction. The absorption at 231.5 mμ was then determined on a Beckman DU spectrophotometer, using pure methanol as a blank.

5. Ribose Alterations

Possible alterations in ribose structure occurring during autoxidation were followed by paper chromatography. Both ascending and descending techniques were employed using Whatman No. 1 paper with butanol : water : acetic acid (250 : 250 : 60) as the developing solvent system. The sugars were detected using 1% $KMnO_4$ in 2% Na_2CO_3. Ribose and ribitol are seen as yellow spots on a purple background with this reagent.

Possible structural alterations in ribose were also studied using polarographic technique. In this work, one-half-gram samples of D-ribose were incubated in air for six days with one gram of methyl linoleate (ML) at room temperature. The reactions mixtures were extracted four times with anhydrous ethyl ether to remove ML and oxidation products. One-tenth-gram samples of the extracted ribose were then dissolved in 100 cc. of phosphate buffers and polarograms were run on 10-ml. aliquots of the stock solution according to the method of Cantor and Peniston. (1)

RESULTS AND DISCUSSION

In earlier work a trypsin assay method was successfully employed to gain information regarding chemical interactions of purified proteins with autoxidizing lipid. As the reaction proceeded it was noted that gelatin foams became increasingly resistant to hydrolytic attack by trypsin. Since this enzyme selectively breaks peptide bonds adjacent to unaltered lysine residues, the hydrolysis data implied that ϵ-amino groups were altered by autoxidizing lipid. This observation was verified by end group marking techniques using insulin-methyl linoleate models.

Because the assay serves as a measure of the final step in the autoxidation of lipids (reaction of cleaved aldehydes with protein amino groups), this method seemed a logical starting point in the screening of potential trapping agents and antioxidants.

In the initial studies, two common hexose and three pentose sugars were incorporated into autoxidizing gelatin-methyl linoleate foams and screened for their protein sparing effect. When the foams were hydrolyzed with trypsin and the liberated nitrogen was analyzed, a considerable variation in antioxidant properties (or in this case, sparing action) of the sugars was noted, as is shown in Table I:

Table I

EFFECT OF SUGARS ON REDUCTION IN HYDROLYSIS RATE IN METHYL LINOLEATE-GELATIN FOAMS

Test Sugar	Percent Reduction in Hydrolysis Rate
No sugar added	22.0
Glucose	19.0
Mannose	7.1
Arabinose	14.1
Xylose	10.5
Ribose	0

As expected from previous work, mannose was shown to have a marked inhibitory effect, but unexpectedly, ribose completely inhibited the gelatin-methyl linoleate interaction. While xylose and arabinose showed some potential, glucose was ineffective as an antioxidant under the conditions employed.

The trypsin method is somewhat tedious, requiring several days for each analysis, and is actually a test for aldehyde trapping agents rather than an index of antioxidant potential. Further screening studies were therefore performed manometrically using reduction in oxygen uptake of purified ML as an index of inhibition. The data in Table II summarizes the results obtained in oxygen uptake studies employing the same sugars which had been screened using the trypsin assay approach.

Table II

INHIBITORY EFFECT OF SUGARS ON OXYGEN UPTAKE OF METHYL LINOLEATE

Sugar	% Inhibition *
Glucose	0
Mannose	28
Arabinose	0
Xylose	0
Ribose	100
Lyxose	78

* Calculated by taking the difference in $\mu l.$ O_2 between control and experimental samples after 180 min. reaction time at 50°C.

Although the data are consistent with those in Table I, a sharper demarcation is apparent. Ribose, lyxose and mannose effectively inhibit oxygen uptake in ML but neither arabinose nor xylose function as antioxidants, as was indicated in the earlier work.

In order to determine whether a structural relationship exists between sugars and their ability to inhibit antioxidation, a number of sugars were screened manometrically, and were found to vary widely in reactivity.

The data given in Figure 1 provide an example of extremes in chemical behavior for two simple sugars with oxidizing methyl linoleate. The three-carbon sugar, glyceraldehyde, appears to function as a pro-oxidant, while the four-carbon sugar, erythrose, is a strong antioxidant. The addition of one CHOH group to the molecule profoundly diminishes the oxygen binding capacity of methyl linoleate under the test conditions. This data plus that obtained with pentoses suggested that the steriochemical configuration about the second carbon atom in the sugar molecule was an important determinant of antioxidant behavior. Ribose, erythrose, lyxose and mannose all contain cis hydroxy groups in the 2 and 3 carbons, while the

unactive sugars do not. Other sugars with cis configurations include D-allose, D-gulose and D-talose. Each of these should function as antioxidants, but unfortunately they were not commercially available for inclusion in this study.

Several sugar derivatives were also screened for potential antioxidant properties in ML systems in order to gain an insight into the reaction mechanisms. The derivatives tested were restricted to those of the most promising sugars, and the data are summarized in Table III.

Table III

THE EFFECT OF SUGAR DERIVATIVES ON METHYL LINOLEATE OXIDATION

Derivative	Rate of O_2 Absorbed (µl. O_2/hour)	Induction Period (hours)
Control	0.09	0.0
Ribitol	0.06	3.4
Erythritol	0.09	0.0
Mannitol	0.09	0.0
Inositol	0.09	0.0
Riboflavin	0.05	1.5
D-2-Deoxyribose	0.01	2.0
Adenosine triphosphate	0.07	0.7

With the exception of ribitol, the reduction of the carbonyl groups appeared to eliminate the antioxidant potency of the sugars. The effectiveness of ribitol may be due to the presence of a ribose contaminant, although no attempt was made to recrystallize the preparation prior to manometric analysis. In addition the data indicate that ribose-containing molecules inhibit oxygen uptake to some degree, and deoxyribose is comparable in antioxidant potency to ribose and erythrose.

On the basis of the data given in Table III, it is tempting to speculate that variability in rancidity in dehydrated meats may hinge on the content of the free riboflavin, A. T. P. and the degree of hydrolysis of DNA and RNA in muscle tissues.

In the screening studies a relatively high concentration of sugar (o. 5 mole) per mole of ML has been used. Since commercial antioxidants are used at much lower levels, ribose at various concentration levels was studied. The data are summarized in Table IV.

Table IV

THE EFFECT OF RIBOSE CONCENTRATION ON METHYL LINOLEATE OXIDATION

Moles of D-Ribose	Molar Ratio ML/Ribose	Rate of O_2 Absorbed (μl. O_2/hour)
0	0	0.095
3×10^{-7}	2000	0.090
3×10^{-6}	200	0.067
3×10^{-5}	20	0.076
3×10^{-4}	2	0.047

The data on ribose concentration do not closely parallel that of phenolic antioxidants. The inhibition of oxygen uptake appears to be directly related to concentration. There is an apparent inversion in the data at the 3×10^{-5} mole level, however, which is reminiscent of the behavior of phenolic antioxidants when they function as prooxidants.

In order to gain an insight into the mechanism of antioxidant action displayed by aldoses, a series of experiments was conducted using diene conjugation as a method for determining at which level in the chain reaction autoxidation is inhibited. In a preliminary experiment using a 1:2 molar ratio of D-ribose to ML, it was noted that the normal 15-minute induction period for ML was extended to four hours upon the addition of this sugar. Diene conjugation was therefore markedly inhibited in the presence of ribose. It would appear that ribose interferes with the autoxidation chain reaction at either the hydrogen atom abstraction stage or it stabilizes the resonance hybrid free atom.

The diene conjugation method was also used to draw a comparison between the antioxidant potency of D-ribose with that of both vitamin E and butylated hydroxyanisole (BHA). Since both phenolic antioxidants absorb some light at the same wavelength used to measure diene conjugation, the method is probably not completely quantitative although appropriate blank corrections are made. It was felt, however, that the method was adequate for drawing rough comparisons of antioxidant potencies.

Data from one such experiment are summarized in Table V.

Table V

ANTIOXIDANT INHIBITION OF DIENE CONJUGATION AS MEASURED BY ABSORPTION AT 231.5 MILLIMICRONS

Antioxidant	Antioxidant Conc. %	Absorption at 231.5 mμ after 7 Hrs. Reaction at 50°C	% Inhibition
Control	0	0.063	0
D-Ribose	0.01	0.019	70
Vitamin E	0.01	0.015	76
BHA	0.01	0.044	30

At a level of .01%, ribose was as effective as vitamin E in inhibiting diene conjugation and it was far superior to BHA.

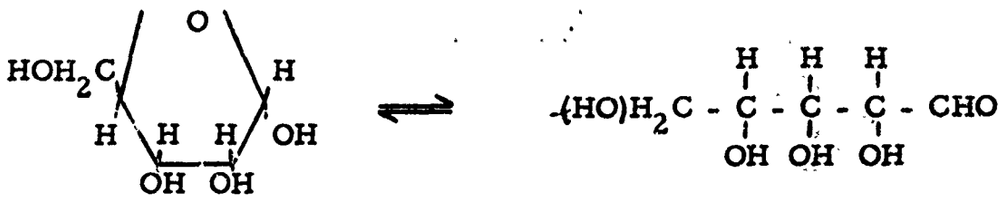
In order to obtain information as to the mechanism by which sugars function as antioxidants a series of paper chromatographic and polarographic experiments was conducted.

In several experiments ribose was extracted away from the autoxidizing ML and then studied using paper chromatography. In all instances a discrete spot for ribose was easily identified, but no other ribose derivative was seen.

It was reasoned that, if ribose reacted stoichiometrically to inhibit autoxidation, this sugar would give up an electron to the linoleate free radicals. If such were the case ribose would be reduced to ribitol and the latter should then be readily identifiable chromatographically. However, since ribitol was not found in extracts from either gelatin-ML foams or from pure mixtures of autoxidizing lipid-ribose mixtures, a reduction reaction is not likely to be involved in the mechanism.

Since the results of the chromatographic approach were uninstructive, polarographic technique was used to determine whether sugars function as antioxidants by virtue of their ability to mutarotate under certain conditions. It was noted that those sugars which display antioxidant behavior, namely mannose and ribose, are those which have a complicated mutarotation and consequently a higher percentage of free aldehydo- form than the average. Ribose solutions, for example, have been shown by Cantor and Peniston (1) to contain appreciable amounts of open chain molecules which could bear on their efficiency as antioxidants.

The reaction which is pH-dependent in aqueous solution may be visualized as follows:



(α-D-Ribofuranose) (Aldehydo-D-Ribose)

It was reasoned that if the aldehydo or reducible form of the sugar molecule was responsible for the antioxidant properties, a shift in equilibrium would occur in the presence of autoxidizing methyl linoleate. Such a shift should be discernible by the polarographic technique reported by Cantor and Peniston. However, when polarograms prepared from ribose which had been incubated with methyl linoleate were compared to pure ribose, the diffusion limiting current remained constant within experimental error. If the ring structure had been opened during autoxidation a much higher diffusion current would have been found.

At this time we have no explanation for the behavior of sugars in the presence of anhydrous ML. The fact that sugars have not been shown to be chemically altered in anhydrous lipid systems under oxidative conditions suggests that perhaps the sparing action is purely a physical effect. It is possible that a molecular alignment occurs between the reactive methylene groups of unsaturated fats and the vicinal C-H groups of the sugar molecules. In aqueous systems where Mabrouk (2) reports them to be prooxidant the hydrated sugars, being hydrophilic, would quite likely be arranged randomly in the aqueous phase of the system and would not complex with the hydrophobic fat globules. This possibility is highly speculative, however, and a great deal of work will be necessary before the dual role of sugars in autoxidizing lipids is understood.

During the course of this investigation dehydrated beef, pork, chicken, and whole milk were fortified with ribose and analyzed both manometrically and with trypsin assays.

Manometric procedures with both beef and milk conducted at 37°C over a 48-hour period failed to show any significant uptake of oxygen in either the controls or ribose samples. Consequently an attempt was then made to extend the trypsin assay technique to the study of antioxidants on dehydrated meat models.

Initially digestion studies were conducted using papain as the test enzyme instead of trypsin, since the latter is not too effective on myosin and other meat proteins. Oddly enough, papain even at relatively high levels was only about half as effective as trypsin, and therefore the latter was used to study meat models. A summary of the data is given in Table VI.

Table VI

LIBERATION OF ALPHA-AMINO NITROGEN IN MEAT-SUGAR DISPERSIONS AFTER AUTOXIDATION AND TRYPSIN HYDROLYSIS

Type of Meat	% Fat	% Reduction in Nitrogen Liberated
Beef	28.6	12.9
Pork	36.5	5.8
Chicken (white)	11.0	8.2
Chicken (dark)	21.0	7.6

From the data in Table VI it can be seen that ribose under the test conditions has little protein sparing effect in dehydrated meats. On the contrary, all samples analyzed showed a diminished amount of nitrogen liberated from ribose-treated samples as compared to dextrose-treated controls. The individual statistics for beef samples ranged from reductions of 19% to as low as 4.5% amino nitrogen, however, and it is therefore unlikely that the trypsin assay data fully depict functions in complex protein mixtures.

It would appear that new experimental procedures, used in conjunction with organoleptic tests, will be necessary in order to properly evaluate the ultimate usefulness of the sugar antioxidants.

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2. Mabrouk, A. F.: J. Am. Oil Chemists' Soc. 41:331, 1964.

Figure 1. The Influence of Simple Sugars on the O_2 Uptake of Methyl Linoleate